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# Scintigraphic detection of xenografted renal tumor by anti-renal cancer monoclonal antibody radiolabeled with technetium-99m

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## SCINTIGRAPHIC DETECTION OF XENOGRAFTED RENAL TUMOR BY ANTI-RENAL CANCER MONOCLONAL ANTIBODY RADIOLABELED WITH TECHNETIUM-99m

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Accumulation in the tumor of the RCS-1 monoclonal antibody, which recognizes the cell surface antigen of renal cancer cells was examined. The antibody purified by affinity chromatography (protein-A column) and gel fractionation was labeled with technetium-99m ( $^{99m}\text{Tc}$ ) by a direct method. High labeling efficiency ( $>98\%$ ) could be routinely obtained. However, the  $^{99m}\text{Tc}$  labeling of the antibody did not reduce the reactivity of the RCS-1 antibody. The labeled antibody was injected into nude mice transplanted with human renal and gastric tumors, and the accumulation of the antibody in each tumor and various tissues was compared at 48 hours after injection. The highest accumulation of radiolabeled RCS-1 antibody was observed in the AM-RC-3 renal tumors; at 8.0% of the injected dose per gram and a tumor-to-blood ratio of 1.05, respectively. However, the radiolabeled RCS-1, did not show specific accumulation in the gastric tumor nor in any tissues tested. The xenografted tumor, AM-RC-3 was successfully visualized with the radiolabeled RCS-1 antibody by scintigraphy.

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**Key words:** RCS-1, Monoclonal antibody, Renal cancer,  $^{99m}\text{Tc}$

### INTRODUCTION

Renal cell carcinoma, occurring only at a prevalence of approximately 1% of all the malignant tumors, is second to bladder cancer and prostate cancer in incidence. Renal cell carcinoma has a poor response to several anti-cancer drugs and neither effective diagnostic procedures nor therapeutic methods have been established. Recently, the expression of drug-resistant genes has been found in renal cell carcinoma and the mechanism of drug resistance has been clarified<sup>1-6</sup>. Therefore, earlier diagnosis, resection of both original and metastatic foci, and elimination of micro-metastatic cancer cells by certain procedures play an important role in improving

therapeutic efficacy and prognosis.

The antibodies to various tumor markers such as  $\alpha$ -fetoprotein<sup>7</sup>, carcinoembryonic antigen<sup>8</sup>, CA19-9<sup>9</sup> and CA125<sup>10</sup>, have provided marked influences on diagnosis and treatment of cancer. To date, the prepared antibodies can be clinically applied for radio-immunoimaging<sup>11,12</sup>. Furthermore, new approaches have been taken for therapy of cancer (Missile therapy) by using intact antibodies or the antibodies conjugated with cytotoxic substances<sup>13</sup>. As specific antibodies to renal cancer cells, S-22 antibody reported by Ueda and Ogata<sup>14</sup>, E6, B7 and C8 by Scharfe and Becht<sup>15</sup>, krc-1, -2, -3 by Tokuyama H and Tokuyama Y<sup>16</sup> and A6H by Vessell et al.<sup>17</sup>, have been documented consequently,

only a few antibodies have been used for the treatment and diagnosis of renal cell carcinoma. Recently, Oosterwijk et al.<sup>18)</sup> reported that the radioimmunoimaging of renal cell carcinoma could be performed relatively specifically.

Our group obtained a monoclonal antibody which specifically reacted with renal cancer cells but did not react with normal renal cells. In this study, we assessed the possibilities of the specific accumulation of RCS-1 antibody in a tumor intending to utilize this antibody for the diagnosis of renal cell cancer.

### MATERIALS AND METHODS

#### 1. Tumor cells

Renal cancer cells (CCF-RC-2)<sup>19)</sup> (provided by Dr. Takayuki Hashimura, Department of Urology, Faculty of Medicine, Kyoto University) reactive with RCS-1 and gastric cancer cells (NUGC-4)<sup>20)</sup> (supplied by Dr. Tadashi Watanabe, Department of Surgery II, Nagoya University School of Medicine) were employed in this study. These cell lines form tumors in nude mice. These cells were cultured in 5% fetal calf serum-RPMI-1640 medium.

#### 2. Tumor-bearing nude mice

In this study, female nude mice [Jcl: MCH (AF)-nu/nu] at ages of 5~8 weeks from Clea Japan Inc., Shizuoka, were used. Nude mice were subcutaneously implanted with human renal cancer cell line, AM-RC-3<sup>21)</sup>, CCF-RC-2 and gastric cancer cell line NUGC-4 to form tumors; incidentally AM-RC-3 was used as the antigen for RCS-1 antibody preparation. Tumors that reached 0.5~1.5 g in weight were used to

assess labeled antibody uptake into tumors.

#### 3. Antibodies

The antibody employed in this study, RCS-1 (IgG1), was specifically reactive with renal cancer cells<sup>22)</sup>. The antibody was purified by protein A affinity chromatography and by gel fractionation by Fast Protein Liquid Chromatography (FPLC, Pharmacia Inc., Sweden) As the negative reference, IgG1 type mouse myeloma protein (MMP, Cappel Inc., PA, USA) produced by mouse myeloma MOPC21 non-reactive with human and mouse tissues was purified in a similar manner.

#### 4. Radiolabeling of antibody

##### A. $^{99m}\text{Tc}$ labeling

Radiolabeling of the antibodies with  $^{99m}\text{Tc}$  was performed according to the method of Mather and Ellison (Fig. 1)<sup>23)</sup>.

To prepare the binding sites for  $^{99m}\text{Tc}$  via cleavage of disulfide bonds in the antibodies, the antibodies were reduced by reaction with a molar excess of 2-mercaptoethanol (2ME) ranging from 1: 500 to 1: 300,000. After treatment with 2ME, the antibodies were divided into 50  $\mu\text{g}$  aliquots and frozen at  $-80^\circ\text{C}$  until radiolabeling.

Upon radiolabeling, one bottle of bone-scanning kit specified for bone disease diagnostic kit (containing 0.381 mg disodium methane-1-hydroxy-1, 1-diphosphonate, 0.190 mg anhydrous stannous chloride, 0.176 mg ascorbic acid and 10.0 mg lactose), Nihon Mediphsix Inc., Hyougo, was reconstituted with 5 ml of physiological saline at room temperature for 5 minutes. Ten  $\mu\text{l}$  of the resultant solution was added to 50  $\mu\text{g}$  of the antibody solution was followed by addition of 0.5 mCi of sodium

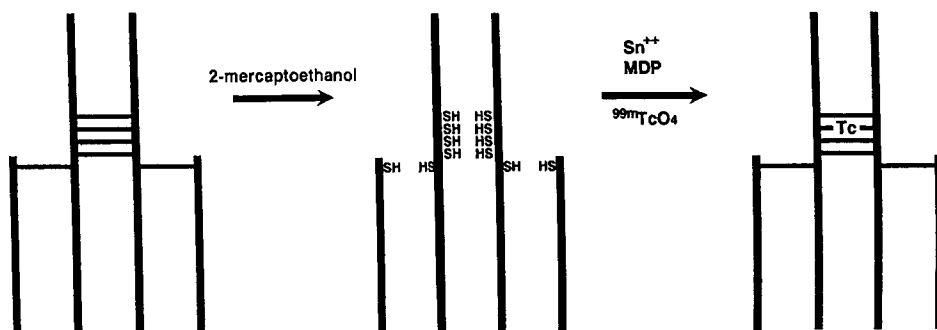


Fig. 1. Scheme showing outline of technetium-99m labeling procedure.

per technetate freshly eluted from a  $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$  generator (Nihon Mediphsix Inc., Hyougo). After being left at room temperature for 15 minutes, the labeled antibodies and free  $^{99\text{m}}\text{Tc}$  were separated by fractionation each into 0.5 ml on PD-10 column using PBS as an eluent. Radioactivity of each fraction was determined by radioisotope calibrator (CRC-30BL, Capintech Inc., PA, USA) to measure labeling efficiency and labeling amount per antibody.

#### B. Activity of labeled antibody

Influences of  $^{99\text{m}}\text{Tc}$  labeling on the antibody activity and its immunoreactivity were examined.

##### a. Influences on antibody activity

The reactivities of the radiolabeled antibodies and non-labeled antibodies against the renal cancer cells (CCF-RC-2) were compared by the MHA method.

##### b. Immunoreactivity of labeled antibodies

In the same manner as indicated above, CCF-RC-2 and NUGC-4 were employed for immunoreactivity of the antibodies. The cells were harvested with 0.02% EDTA (ethylenediamine-tetraacetic acid) - PBS and washed with the cell culture medium. Into a 1.5 ml microtube,  $5 \times 10^6$  cells or  $1 \times 10^6$  cells was inoculated followed by centrifugation for 5 minutes at 3,000 rpm and the supernatant was discarded. The radio-labeled antibodies adjusted with the cell culture medium to make  $0.5 \mu\text{Ci}/50 \mu\text{l}$  were added to these cells and the mixture was allowed to react for 2 hours on ice water. After completion of the reaction, the cells were centrifuged for 5 minutes at 12,000 rpm. The radioactivity in the supernatant and the cell pellets were measured by a Gamma-counter (LKB-Wallac Clinigamma 1272, LKB Inc., Tuusula, Finland). The binding ratio of the antibody bound to cells was calculated from the values (cpm) of the supernatant and the cell pellet.

#### C. Accumulation of antibodies

Biodistribution of the labeled antibodies and intratumor uptake were determined using  $^{99\text{m}}\text{Tc}$ -labeling of RCS-1 antibody and MMP as the negative reference.

$^{99\text{m}}\text{Tc}$ -labeled antibodies at  $0.5 \mu\text{Ci}$  per

$200 \mu\text{l}$  of PBS for observation 24 and 48 hours after injection, and at  $8.0 \mu\text{Ci}$  per  $200 \mu\text{l}$  of PBS for observation 72 hours after injection were intra-peritoneally injected into tumor-bearing and control nude mice using a syringe for insulin injection. The labeled antibody solution at the same volume as the dose for mice was aliquoted into a scintillation tube and subjected to determination of the labeled antibody amount at the respective determination times.

Blood was collected from the heart of injected mice under ether anesthesia, and the organs from the mice were weighed together with determination of radioactivity. Digestive tracts were weighed after elimination of their contents, followed by determination of radioactivity. Accumulation ratios to organs and tumors were calculated according to the equation as outlined in the footnote to Table 2 by converting the mouse weight to 20 g. Intratumor uptake of the antibodies was imaged under the supervision of Dr. Harumi Sakahara, Department of Nuclear Medicine, Faculty of Medicine, Kyoto University.

To determine the accumulation ratio of the labeled antibodies in the present study, renal cancer cell AM-RC-3 having a tumor-to-blood ratio over 1.0 was used. The labeled antibody  $314 \mu\text{Ci}/200 \mu\text{l}$  was injected into a tail vein and 24 hours later, mice were anesthetized by intraperitoneal injection of 0.5 ml of 10-fold diluted Nembutal (Dainabot Co., Ltd., IL, USA) with physiological saline. Subsequently, mice were imaged on a gamma camera equipped with a pinhole collimator (Searle PHO/GAMMA LFOV, Searle Radiographics Inc., Des Plaines, IL, USA).

#### D. Reactivity of the antibody

Reactivity of the antibody was analyzed by the MHA method<sup>24)</sup> essentially the same as previously described<sup>25)</sup>.

## RESULTS

#### 1. $^{99\text{m}}\text{Tc}$ labeling of antibodies

By varying the mixing ratio of the antibodies and 2ME ranging from 1: 500 to 1:300,000, the effect of 2ME treatment to

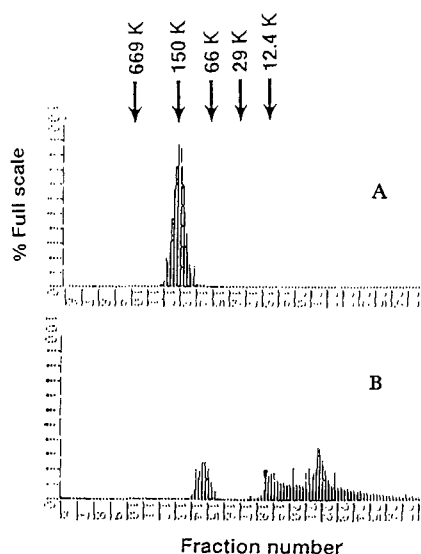


Fig. 2. Diagram of protein detector profile on FPLC analysis of antibody solution. Chromatography using Superdex 200HR 10/30 was performed as described in "Materials and Methods". Fractions were monitored for protein by absorbance of light at 280 nm. A. Chromatography of RCS-1 antibody reduced with 2ME (reducing ratio of 1: 2,000). Only one peak containing RCS-1 antibody was observed. B. Chromatography of RCS-1 antibody reduced with 2ME (reducing ratio of 1: 300,000). Some peaks (antibody fragments) which were made by reducing the antibody were observed.

antibodies was examined on the antibody molecule-structure, antibody immunoreactivity, and the labeling efficiency of  $^{99m}\text{Tc}$  were examined by counting  $^{99m}\text{Tc}$  in the labeled antibodies and free  $^{99m}\text{Tc}$  following fractionation with gel filtration.

Following the reaction with a molar excess of 2ME ranging from 1: 500 to 1: 8,000, the antibodies were eluted into the same fraction as the intact antibodies (Fig. 2-A) while at a ratio of 1: 300,000, the disulfide bonds in the antibodies were cleaved and the original peak due to the intact antibodies disappeared in parallel with the occurrence of several peaks attributable to proteins with smaller molecular weights than the original antibodies (Fig. 2-B), indicating 2ME-induced degradation of the antibody molecule. With

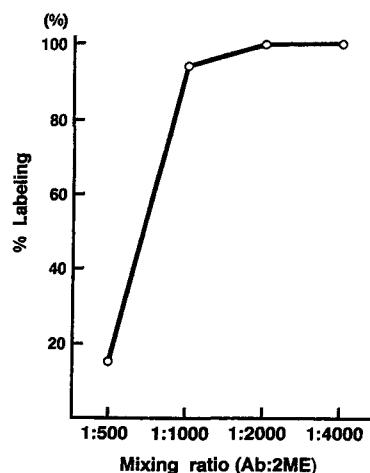


Fig. 3. Effect of reduction conditions upon antibody labelling efficiency.

application of 2ME at a ratio of 1: 500~8,000, the reactivity was equivalent to that of the intact antibodies, indicating that the antibody immunoreactivity was resistant to this treatment with 2ME. Therefore, we confirmed that the reactivity of antibody was retained within the ranges applied for labeling in this study.

As illustrated in Fig. 3, the labeling efficiency accounted for approximately 15% at the antibody: 2ME ratio of 1: 500, but the corresponding efficiencies at 1: 1,000 and 1: 2,000 were 94% and 99%, respectively. At the mixing ratio of 1: 2,000, the labeled amount of  $^{99m}\text{Tc}$  uptaken the antibodies was roughly  $10 \mu\text{Ci}/\mu\text{g}$ , which corroborated that the disulfide bonds were well cleaved to permit almost complete incorporation of  $^{99m}\text{Tc}$  into the labeling.

## 2. Activity of labeled antibody

The antibody activities before and after labeling by the MHA method revealed no difference between the unlabeled and labeled antibodies. To assess the specificity of the reaction, renal cancer cells (CCF-RC-2) and gastric cancer cells (NUGC-4) were allowed to react with the labeled antibodies and comparisons were made between the bound radioactivities and the unbound radioactivities in the supernatant. As Fig. 4 shows, in the experiment using  $5 \times 10^6$  cells of renal cancer cells, about 50 % of the labeled antibodies reacted with

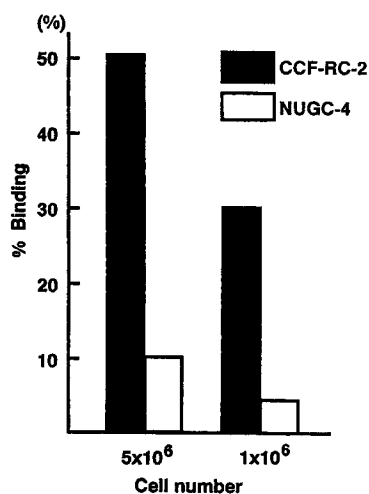


Fig. 4. Reactivities of <sup>99m</sup>Tc-labeled RCS-1 antibody to renal cancer cells (CCF-RC-2) and gastric cancer cells (NUGC-4).

cells while about 30% of the labeled antibodies reacted with  $1 \times 10^6$  cells. By contrast, the corresponding percentages in the experiment using  $5 \times 10^6$  and  $1 \times 10^6$  cells of gastric cancer cells were 11 and 5%, respectively. These findings suggested that the specific reactivity of this antibody could be well retained even with <sup>99m</sup>Tc labeling.

### 3. Biodistribution of labeled antibodies

The objectives of this study were to assess 1) how and to what extent the labeled antibodies are distributed after intraperitoneal administration, 2) when observation should be performed after administration, 3) whether accumulation into tumors really occur, and 4) whether its accumulation is associated with the reaction specificity.

The time-course changes of biodistribution of the injected antibodies were monitored in nude mice carrying human renal cancer cells, AM-RC-3. Fig. 5 shows the radioactivities (% dose/g) distributed to each gram of the respective organs. At 24 hours after injection, about 10 and 8.5% of injected antibody were distributed to the blood and kidneys, respectively. Distribution to tumors, on the other hand, was as low as 6.5%. However, at 48 hours after injection, the uptake into the blood and kidneys was reduced to about 7.5 and

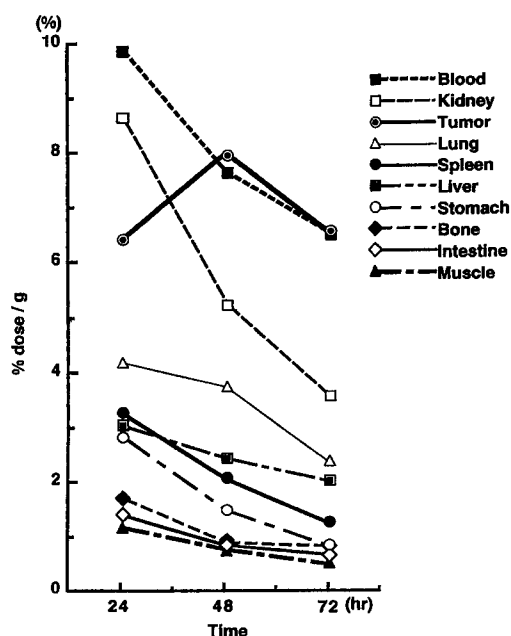


Fig. 5 Biodistribution of <sup>99m</sup>Tc-labeled RCS-1 antibody in the AM-RC-3 tumor bearing mice at 24, 48, and 72 hr postinjection.

5%, respectively, while the distribution to the tumor was obviously increased to approximately 8%. The distribution ratio of blood and kidney/tumor was reversed by increased uptake in the tumor, indicating a marked accumulation of the radio-labeled antibody in the tumor. At 72 hours after injection, the tendency was similar to that observed at 48 hours post administration, was evidenced suggesting that observation of intratumor uptake becomes feasible at 48~72 hours after intraperitoneal injection. Based on these findings, the following experiments were conducted by the observation at 48 hours after administration.

Accumulation of labeled antibody in tumor was examined using MMP and AM-RC-3 tumors. As Table 1 shows, the intratumor accumulation of RCS-1 antibody and MMP was 8% and 3.68%, respectively indicating more significant uptake of RCS-1 antibody in the tumor ( $P < 0.01$ ) than of MMP. Similar findings were also obtained on the biodistribution of MMP in organs of intact nude mice and the

Table 1. Biodistribution of  $^{99m}\text{Tc}$ -labeled RCS-1 and MMP in AM-RC-3 bearing nude mice, and MMP in tumor-free nude mice on day 2

Tumor antibody	AM-RC-3 (N=3) RCS-1	AM-RC-3 (N=3) MMP	—(N=3) MMP
% dose / g <sup>a)</sup>			
Tumor	8.00 (6.70–9.21) <sup>b)</sup>	3.68 (3.23–4.21)	—
Blood	7.65 (6.02–8.79)	8.54 (7.00–9.49)	8.44 (6.37– 9.71)
Liver	2.46 (2.14–2.64)	2.24 (1.69–3.08)	2.61 (1.87– 3.26)
Kidney	5.26 (4.99–5.43)	5.07 (4.19–6.58)	6.31 (5.04–13.17)
Intestine	0.82 (0.70–0.92)	0.84 (0.60–1.02)	0.74 (0.42– 0.98)
Stomach	1.50 (1.04–1.78)	2.23 (1.69–3.02)	2.88 (2.07– 3.30)
Spleen	2.06 (0.93–2.99)	1.88 (0.98–2.71)	2.31 (1.62– 2.85)
Lung	3.73 (3.09–4.36)	4.37 (3.34–5.13)	5.21 (3.55– 6.08)
Muscle	0.79 (0.71–0.89)	0.77 (0.65–0.98)	0.65 (0.20– 0.95)
Bone	0.94 (0.38–1.26)	1.02 (0.51–1.89)	1.1 (0.715– 1.55)
Tumor / Blood <sup>c)</sup>	1.05 (0.92–1.13)	0.40 (0.24–0.49)	—

a) Distribution data were expressed as a percentage of the injected dose per gram of tissue normalized to a 20 g mouse.

A=Amount (cpm) of organic isotope

B=Weight (g) of an organ

C=Weight (g) of a nude mouse

T=Amount (cpm) of injected isotope

$$\% \text{ Dose/g} = \frac{A}{T \times B} \times 100 \times \frac{C}{20}$$

b) Mean and range

c) Tumor to blood ratio

Table 2. Biodistribution of  $^{99m}\text{Tc}$ -labeled RCS-1 in tumor-bearing mice on day 2

Tissue	Tumor		
	CCF-RC-2 (N=3)	AM-RC-3 (N=3)	NUGC-4 (N=3)
%dose / g			
Tumor	5.43 (5.40–5.45) <sup>a)</sup>	8.00 (6.70–9.21)	4.92 (3.95– 6.05)
Blood	6.81 (6.29–7.66)	7.65 (6.02–8.79)	8.85 (6.90–10.19)
Tumor / Blood <sup>b)</sup>	0.80 (0.71–0.86)	1.05 (0.92–1.13)	0.55 (0.50– 0.57)

a) Mean and range

b) Tumor to blood ratio

AM-RC-3 bearing nude mice. In the nude mice transplanted with renal tumor, accumulation of RCS-1 antibody was smaller in any organs other than the tumor. Therefore, these findings corroborated that RCS-1 antibody was not specifically accumulated in any organs other than the tumor.

Accumulation specificity was examined using AM-RC-3, CCF-RC-2 and NUGC-4. Accumulation of the antibody in respective tumors was compared by % dose/g and the tumor-to-blood ratio. The % dose/g of NUGC-4, CCF-RC-2 and AM-RC-3 was 4.92, 5.43 and 8.0%, respectively,

suggesting higher accumulation into the tumor reactive with the antibody (Table 2). The tumor-to-blood ratio as an index representing intratumor accumulation was 0.55, 0.80 and 1.05, respectively, confirming that antibody specificity resulted in a significant by higher accumulation ( $P < 0.01$ ) in renal cancer cells than in gastric cancer.

Fig. 6 shows an image of the radio-labeled RCS-1 antibody accumulation in AM-RC-3. The chest and abdomen of mice showed a moderate accumulation, and the tumor demonstrated antibody uptake providing, visual confirmation of the antibody accumulation therein.

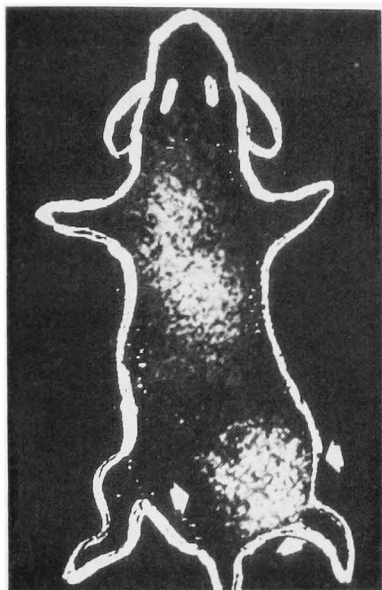


Fig. 6. Scintigram of a nude mouse bearing renal tumor (AM-RC-3). Scintigram was taken at 24 hr after injection of  $^{99m}\text{Tc}$ -labeled RCS-1 antibody. Arrow indicates bearing tumor position.

### DISCUSSION

The  $^{99m}\text{Tc}$ -labeling method employed in the present study is called the direct method but its labeling mechanism remains to be elucidated. However, disulfide bridges within the antibody are said to be cleaved by the reductant 2ME, followed by insertion of  $^{99m}\text{Tc}$  and its binding to SH. The unbound SH groups are easily oxidized to form disulfide bonds, with less influence on antibody activities. Mather and Ellison<sup>23)</sup> examined these profiles and succeeded in labeling their antibodies without lowering their activities. Various antibodies are known to show different sensitivities to chemical modifications. We examined whether radio-labeling could be achieved without affecting RCS-1 antibody activities and whether the labeled antibody could be specifically accumulated in tumors. Furthermore, FPLC, performed to determine whether cleavage of disulfide bonds occurs without affecting the antibody activities revealed no abnormality in the molecular size of the antibody. After treatment with 2ME at a molecular ratio

of 1: 500~1: 8,000, the antibody was not cleaved so as to form separate fragments of H and L chains, suggesting that disulfide bonds in the antibody were partially cleaved but the antibody molecule retained the original conformation. However, some concerns about influences on the reactivity were raised because disulfide bonds are located in the bonds between H and L chains, the bonds between H chains, and the part connecting with the antigen; therefore the influence with 2ME-treatment on reactivity was examined. The reactivity of the treated antibodies was not different from that of the intact antibodies, and at a molar ratio of 1: 500~1: 8,000 (Antibody: 2ME), the reactivity of the antibodies was not affected.  $^{99m}\text{Tc}$ -labeling reached a plateau at a molar ratio of 1: 2,000 and the labeling efficacy accounted for 99%, which was slightly different from the findings of Mather and Ellison. No differences were noted in the reactivity and specificity between  $^{99m}\text{Tc}$ -labeled antibody and the antibody which was similarly processed except for the addition of  $^{99m}\text{Tc}$  in the reaction mixture.

To determine the most appropriate observation time for accumulation of the labeled RCS-1 antibody, time-course observations were performed in nude mice bearing the renal cancer cells, AM-RC-3. At 24 hours after injection, the labeled antibody was predominantly observed in the blood, followed by the kidney as the supposed excretion route. However, at 48 hours after injection, radioactivity was decreased, particularly in the kidney. Accumulation of the antibody into the tumor was less evident than that in blood and kidneys at 24 hours after injection, but the accumulation ratio was higher than that in the blood and kidneys at 48 and 72 hours after injection. Other organs demonstrated little accumulation of the antibody observed with fewer time-course changes. These findings indicated that the observation for antibodies should be made at 48 hours or later after intraperitoneal injection.

As shown in Table 1, accumulation of MMP in organs other than the tumor cells



showed no difference between tumor-bearing nude mice and the control nude mice. However, the accumulation of RCS-1 antibody and MMP in tumors expressed as % dose/g was 8% for RCS-1 and 3.68% for MMP, indicating an obvious difference. Thus RCS-1 antibody was specifically accumulated in several renal cancer cells but not into any organs other than the tumor cells.

As illustrated in Fig. 6, accumulation of RI was confirmed in the tumor (arrow) while the labeled antibody was also accumulated in the liver and the kidneys.

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## 和文抄録

### テクネシウム標識した抗腎癌モノクローナル抗体による 移植腎腫瘍でのシンチグラムの試み

愛知医科大学泌尿器科学教室 (主任: 深津英捷教授)

三井 健司, 瀧 知弘, 宮川 嘉眞  
山田 芳彰, 本多 靖明, 深津 英捷

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腎細胞癌の画像診断をめざして, 腎細胞癌に特異的に反応するモノクローナル抗体 (RCS-1) の, 生体内での腫瘍への集積性の基礎的検討を行った. 抗体はテクネシウム標識して検査に用いた. 標識抗体の腫瘍への集積性は, 腎細胞癌 (AM-RC-3, CCF-RC-2), 胃癌 (NUGC-4) 移植ヌードマウスを用いて検討した. 標識抗体を腹腔に投与し, 48時間後に各組織への集積を検査した. 腫瘍への集積性の指標となる腫瘍/血液で各腫瘍を比較するとそれぞれ1.05, 0.80, 0.55とな

り胃癌株に対して腎細胞癌株に有意に集積しており, 腎細胞癌特異的な反応として集積していることが確認できた. またシンチグラム像としても本抗体の集積を確認することができた. 以上より抗腎細胞癌モノクローナル抗体 (RCS-1) が生体内で特異的に腎腫瘍に集積することが観察され, 画像診断への応用の可能性を示唆することができた.

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